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A UPLC-MS/MS Method for Therapeutic Drug Monitoring of Etonogestrel

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Abstract

Introduction—Etonogestrel is a progestin used in the contraceptive vaginal ring NuvaRing and the subdermal implant Implanon. A sensitive method for measuring etonogestrel is useful for further investigating the progestin's pharmacokinetics with these alternative contraceptive formulations as well as generating important information about possible continued efficacy or potential failure to remove the subdermal implant.

Methods—Standards and serum samples were spiked with D8 progesterone (internal standard) and subsequently extracted with dichloromethane, dried, and reconstituted in 25% methanol with formic acid. Etonogestrel was analyzed by positive electrospray ionization in multiple reaction monitoring mode with a run time of 5.5 minutes using a C18 BEH column. The mobile phase was a gradient of water:acetonitrile, with 0.1% formic acid. The method was applied successfully to study the pharmacokinetics of etonogestrel during vaginal ring use. The method was also used in routine patient care to assess etonogestrel levels.

Results—The method is linear from 50pg/ml to 2000pg/ml. The limit of detection and quantification (LOD and LOQ) are 25 and 50pg/ml respectively. There was no observed ionization suppression within the linear range of the assay and the average recovery was 87%. Serum etonogestrel levels of n=3 subjects were all within the linear range of the assay for a total study period of 42 days after insertion of the ring. Of n=20 patients with non-palpable subdermal implants, n=13 had etonogestrel levels > 25 pg/mL, while n=7 had levels < 25 pg/mL.

Conclusions—We developed a rapid, sensitive, and robust UPLC-MS/MS method for the quantification of etonogestrel in serum that is useful to study the progestin's pharmacokinetics and inform physicians about successful implantation or potential failure to remove a subdermal device.

Keywords

Etonogestrel; LCMSMS; contraception; pharmacokinetics; vaginal ring

Introduction

Hormonal contraceptive methods are effective at preventing pregnancy. They can consist of a single hormone or combinations of hormones such as a progestin and an estrogen. The drugs are usually taken orally but alternative routes of administration are available and

provide distinct advantages such as more convenient dosing regimens, improved adherence, lower dosages relative to oral use and more stable levels of the hormones [1–3]. Alternative routes of administration circumvent oral absorption, which is characterized by substantial variability in pharmacokinetics and pharmacodynamics [4]. These delivery approaches include intramuscular, transdermal, subdermal and vaginal administration.

Although the development of a contraceptive vaginal ring began as early as the 1960s [5], today the NuvaRing is the only ring contraceptive available to the United States market [4, 6]. Introduced in 2001, the efficacy and tolerability of this ring, which releases 15 µg of ethinyl estradiol (EE2) and 120 µg of etonogestrel (ENG) per day for a period of at least 3 weeks, has been demonstrated in a number of studies [1, 7, 8]. Use of ring and implant based contraception in the United States and globally is growing. Increased access to highly effective and longer acting methods such as Implanon® and the NuvaRing® is a crucial area to address the globally unmet needs for contraception[9].

Any contraceptive drug has the chance of therapeutic failure [10]. There are multiple potential reasons for an increased failure rate such as nonadherence, food and drug interactions as well as under-dosing, all leading to decreased systemic exposure to the hormones [11–13]. While the combined EE2/ENG vaginal ring is theoretically less prone to food and drug interactions than oral anti-contraceptives, the pharmacokinetics of EE2/ENG can be altered by other drugs and food or patient-related factors such body weight, potentially leading to sub-therapeutic levels of the hormones and failure of anti-conceptive therapy. However, the number of pharmacokinetic studies with the vaginal ring exploring the role of pharmacokinetics in therapeutic failure is limited.

Etonogestrel, which is the active metabolite of desogestrel, a progestin that has been used in oral contraceptives for many years, is also administered by placing an implant (Implanon, Merck, USA) subdermally that slowly releases the progestin for a prolonged period of time. During the first five to six weeks after insertion, etonogestrel is released at a rate of 60–70 microgram per day. This rate decreases to 35–45 µg per day 1 year, 30–40 µg/day two years, and 25–30 µg/day three years after implantation of the device [14]. It is recommended that the device is removed after 3 years in non-obese, and possibly after 2 years in obese women. Usually the device can be located by feeling through the skin but sometimes this is not possible, in which case presence of the device needs to be confirmed by X-rays, CT, echography or MRI. Presence or absence of the device is also determined by assessing an etonogestrel level in serum; however, this option has not been widely available because a non-proprietary sufficiently sensitive assay for etonogestrel did not exist.

We therefore developed and validated a sensitive LC-MS/MS assay to measure etonogestrel in serum. The assay can be used to further investigate the pharmacokinetics of the progestin as well as to confirm the presence or absence of a subdermal device.

Methods

Chemicals

Etonogestrel was a generous gift from Merck (Merck, USA). D8-Progesterone (DLM-6909-1.2; Internal standard) was purchased from Cambridge Isotope Laboratories (Andover, MA). Dichloromethane, Formic Acid, Water and Acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA) and LC-MS grade if possible. Stripped human serum (MSG3000) was purchased from Golden West Biologicals Inc. (Temecula, CA).

Standards

Etonogestrel was dissolved in methanol and used as the parent stock solution. Working stock solutions of 10, 1, 0.1, and 0.01ng/ml were made in methanol and were stored at -80°C . Stock D8-progesterone was diluted in methanol to a 10ng/ml working stock solution.

Sample Processing

All samples were stored at -80°C prior to analysis. 250 μL of serum was used for quantification of etonogestrel. 250pg of the internal standard (D8-progesterone) was added to each sample or standard followed by extraction with 6ml of dichlormethane. The samples were vortexed for 10 minutes, centrifuged and the supernatant collected, dried under nitrogen and reconstituted in 100 μL 25% methanol containing 0.1% formic acid.

Liquid Chromatography

A BEH C18 Acquity UPLC column (2.1 $\times$ 50mm, 1.7 μm particle size) (Waters Corp, Milford, MA) was used on a Waters Acquity UPLC. The mobile phase included H_2O (A) and Acetonitrile (B) both with 0.1% formic acid. The gradient began with 30% B and increased linearly to 95% B over 4.75 minutes. Initial conditions were re-achieved by 4.9 minutes and maintained for re-equilibration until the end of the run. Total run time was 5.5 minutes and the flow rate was 0.5ml/min.

Mass Spectrometry

Etonogestrel was quantified by positive electrospray ionization using the Waters Xevo TQ-S system (Waters Corp, Milford, MA). System conditions were optimized to maximize the signal. Conditions were as follows: Capillary Voltage=2.9kV, Cone Voltage 38V, Desolvation Temp = 500C, Desolvation Flow = 500 l/hr, and Cone Flow = 150 l/hr. Standards and samples were run in multiple reaction monitoring (MRM) of the H^+ ion with the transitions 352.2 to 109.1 (collision energy = 26V) for quantification in addition to 352.2 to 147.1 (collision energy = 22V) for confirmation of peak purity. The internal standard, D8-progesterone, had an optimal cone voltage of 42V with the following transition: 323.3 to 100.1 (collision energy = 22V).

Sample Quantification

A 7-point calibration curve was made by spiking 250 μL of serum with etonogestrel. Relative response to the IS was plotted on a linear standard curve with 1/X weighting.

Ion Suppression/Enhancement

Ionization suppression/enhancement experiments were performed using pooled serum from 2 patients that tested negative ($< 25\text{pg/ml}$) for etonogestrel. For each level, 250 μL of etonogestrel negative serum was extracted as above. The extracted solution was spiked with 12.5, 25, 62.5, 125, and 250pg corresponding to final concentrations of 50, 100, 250, 500, and 1000pg/ml respectively. Responses of the extracted then spiked samples were compared to responses of the standard in neat 25% MeOH. Ion suppression/enhancement was calculated as follows: $[(\text{response in matrix})/(\text{response in MeOH})]*100$.

Extraction Efficiency

As per the Ionization suppression/enhancement experiments, extraction efficiency was performed using pooled serum from 2 patients that tested negative ($< 25\text{pg/ml}$) for etonogestrol using the same levels as above. The negative serum was spiked prior to isolation. The response was compared to samples that were spiked after negative serum was

extracted (i.e. extraction efficiency is derived as the ratio of responses obtained from the spiked then extracted samples to the samples that were extracted then spiked).

Interference

Interference from lipids and hemoglobin was investigated by comparing Etonogestrel (at 350 and 1500 pg/mL) and Internal Standard peak areas in samples with increasing concentrations of lipids (Intralipid® 125; 250; 500;1000 mg/dL, n=4 samples at each concentration) and increasing concentrations of hemoglobin (188; 275; 750 mg/dL, n=4 samples at each concentration).

Stability

Stability was investigated for three levels of QC samples (75, 750, 1500 pg/mL; n = 4) during storage for 72 h at 4°C and 90 days at -80°C.

Lower Limits of Detection, Quantification

Using pooled serum that tested negative for etonogestrel (< 25pg/ml) we determined the limits detection and quantification by spiking various amounts of etonogestrel into the matrix. The signal to noise ratio was calculated by the Waters data analysis software and was the average of 4 – 5 replicates. The lower limit of detection (LOD) for each species was defined as the concentration at which the signal-to-noise (S/N) ratio is at least equal to 3. Similarly, our lower limit of quantification (LOQ) was defined as the concentration giving a S/N ratio of at least 10. S/N was calculated using a peak-to-peak method in TargetLynx software assigning the average noise level to the area immediately preceding the etonogestrel peak.

Linearity, Accuracy and Precision

Linearity was assessed from 50pg/ml to 2000pg/ml on 5 different calibration curves repeated on different days. Intra-assay variability was assessed by extracting and running 5 levels of controls (50pg/ml, 100pg/ml, 250pg/ml, 500pg/ml, and 1000pg/ml) a total of 4–5 times on the same day. Inter-assay imprecision was assessed by running three quality controls (75pg/ml, 750pg/ml and 1750pg/ml) over 8 days. Accuracy was assessed by the calculated concentration of the quality controls run over 7 days relative to the predicted concentration.

Patient samples

Serum from n=3 subjects using a contraceptive vaginal ring with EE2 and ENG (Nuvaring, Merck, USA) were collected biweekly for 6 weeks (n=12 samples/subject). Etonogestrel levels were also assessed in n=20 serum samples from Implanon (Merck, USA) users that were sent to our laboratory on dry ice for routine patient care.

Sample procurement for the pharmacokinetic study and use of the serum concentrations measured for patient care were authorized by the Institutional Review Board at Columbia University Medical Center.

Results

Chromatography

Etonogestrel has good affinity to the C18 BEH Acquity UPLC column using a water/acetonitrile gradient (retention time = 1.8 minutes; void volume ~ 0.5 minutes). The internal standard elutes at 2.1 minutes. No carryover or interference from previous samples was observed when the gradient was extended to 95% acetonitrile.

Recovery, ionization suppression and interference

We observed modest ionization enhancement at the lowest quantifiable concentration (50pg/ml), but no matrix effect at concentrations greater than 100pg/ml (table 1). The qualifying ion ratio was 1.5. Samples that deviated by more than 20% were reanalyzed at a later date. There was no significant interference from either intralipid or hemoglobin. Peak areas in samples with intralipid or hemoglobin were all within 90–110% of those without intralipid or hemoglobin.

Stability

Etonogestrel was stable in serum during 72 hours at 22°C and during 90 days at –80°C. Concentrations at 24, 48, 72h and 90 days were all within 90–110% of the initial concentration.

Limits of Detection and Quantification

We determined that 25pg/ml and 50pg/ml are the limits of detection and quantification respectively (table 1). Though 25pg/ml has a S/N of ~ 9, we conservatively chose 50pg/ml as our limit of quantification because the signal for the qualifying ion was too low resulting in a skewed qualifying ion ratio.

Linearity, Accuracy and Precision

The correlation coefficient (R^2) of 5 calibration lines repeated on different days was >0.99 using a linear regression model (figure 1). Accuracy was greater than 90% for our 3 QC's (75, 750, and 1750pg/ml).

Precision results are summarized in tables 1 and 2. Intra-assay variability was less than 6% for all concentrations and inter-assay variability was less than 9 – 13% for the 3 levels investigated.

Patient Samples

Etonogestrel concentrations were determined in serum samples collected over the course of 42 days from n=3 subjects using the Nuvaring. The subjects ranged in age from 23 to 35 years old and were normal body weight (BMI < 25). After 2 days of NuvaRing use, serum etonogestrel concentrations were between 1000 and 1500pg/ml. The subjects maintained steady state levels over the 42 day study period (figure 2).

Etonogestrel was not detectable (< 25 pg/mL) in n=7 out of n=20 randomly selected patient samples that were sent to our laboratory for etonogestrel levels due to a non-palpable implant by the referring physicians. Etonogestrel was detected in the remaining n=13 samples with a mean ($\pm$ SD) concentration of 378 ($\pm$ 220) pg/mL and a range of 126–715 pg/mL.

Discussion

We here describe a rapid, sensitive, and robust method for measuring etonogestrel in human serum. The method employs a simple liquid/liquid extraction scheme that is both effective (isolation efficiency greater than 83%) and efficient allowing throughput of ~ 70 samples per day. Based strictly on the signal to noise ratio, the limit of quantification is between 25 and 50pg/ml. Further, linearity extends down to 25pg/ml. We decided to set a more conservative threshold for the limits of quantification to allow for sufficient signal for the qualifying ion to consistently meet the qualifying ion ratio of 1.5. The signal to noise that we established for etonogestrel (50pg/ml) consistently shows S/N greater than 25. The

sensitivity of this assay compares favorably to the noncommercially available RIA (LOQ ~ 30pg/ml) described earlier in the literature[2] as well as to a recently described non-proprietary LC-MS/MS method (LOQ ~ 1ng/mL)[15].

Various potential methodologies are available to measure etonogestrel in serum samples such as High Performance Liquid Chromatography (HPLC), Gas Chromatography-Mass Spectrometry (GC-MS) and LC-MS/MS. We decided to develop a method using the latter platform based on throughput and sensitivity. LC-MS/MS based steroid analysis using current high end instrumentation is often equally as sensitive to GC-MS based analysis without requiring any derivatization. Additionally, the runtimes of LC-MS/MS are significantly shorter than GC-MS based assays. Indeed, in our assay, etonogestrel has a retention time of 1.8 minutes. This is far beyond the void volume, which is around 0.5 minutes. Faster run times using the Acquity UPLC are conceivably possible, but we wanted to make sure that we ran a gradient that was organic enough to eliminate carryover of interfering peaks from previous injections. We found that a gradient ending in 95% acetonitrile over 4.75 minutes was associated with no carryover from the previous samples even at the highest concentrations. Further, this UPLC method allows a throughput of ~ 250 samples per day. We are currently only able to isolate 70 samples per day using our liquid/liquid isolation procedure. Even if we were able to automate the isolation scheme to double our throughput, our inlet method would still be sufficient for our needs.

We found minimal ionization enhancement at the limit of quantification, but no matrix effect at concentrations greater than 100pg/ml. There was also minimal interference from Intralipid and hemoglobin suggesting little influence from lipolysis or hemolysis. The method is therefore void of substantial interference.

At the time of development of this assay, deuterium-labeled etonogestrel was not commercially available so D8-progesterone was used as the internal standard. This is not a perfect solution because the two molecules have slightly different retention times (1.8 minutes vs. 2.1 minutes) so they can conceivably ionize differently if there is a matrix effect. However, we showed no matrix effect at concentrations greater than 100pg/ml with etonogestrel. Deuterated etonogestrel has since become commercially available.

We were able to test our UPLC-MS/MS method on n=3 subjects using the combined EE2/ENG vaginal ring during an observation period of 6 weeks. The ring is supposed to continuously deliver 120ug/day of ENG over a period of 3 weeks, and has been described to continue to deliver ENG for another 3 weeks [2]. The use of the ring quickly increased serum ENG levels to 1000 to 1500pg/ml and reached steady state levels within the first week for all three subjects. These levels up to three weeks after insertion of the ring were confirmed in a recently published prospective clinical pharmacokinetic study using our UPLC-MS/MS method [16]. Interestingly, in the three women we studied for the validation of our assay, steady state etonogestrel levels were maintained for the entire 42 day study period. These data are in agreement with earlier data on ENG pharmacokinetics in women using the combined EE2/ENG vaginal ring [2]. In this study, which used a radioimmunoassay to measure ENG, average levels were also around 1500 pg/mL. Therefore, although we did not compare the methods directly, these data clearly suggest that our UPLC-MS/MS method gives similar results as the earlier described RIA.

Our data also demonstrate that the method can be applied to determine if a subdermal device is (still) in place. While serum concentrations are higher immediately after insertion (average peak concentration of 813pg/ml is reached after 4 days post insertion), average long-term use levels range between 111 and 260 pg/mL[14]. An etonogestrel level in this range or higher therefore suggests that the device was implanted successfully. The mean

half-life of etonogestrel with use of the Implanon device is 25 hours. After implant removal, Etonogestrel levels become undetectable after a mean of 6 days (range 1–10) with Implanon [14]. A detectable etonogestrel concentration in a sample collected at least ten days after alleged removal of the device could therefore mean that the device is still in place. A non-detectable etonogestrel level in such a sample suggests that the device was removed successfully or had never been successfully inserted. However, it could also mean that the device is still in place but releases only very low levels of etonogestrel in the case of a faulty implant.

In conclusion we describe a rapid, sensitive, and robust method for measuring etonogestrel in serum. The assay does not require complicated SPE isolation schemes, the use of radioactivity, or derivatization. The assay is used in clinical studies as well as therapeutic drug monitoring for patient care.

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Figure 1.
Representative calibration line for Etonogestrel in human serum (50–2000 pg/mL). $R^2 = 0.99$.

Figure 2. Etonogestrel serum concentrations in n=3 patients using the Nuvaring intravaginal device.

Table 1

Concentration, extraction recovery, ion-enhancement, precision and signal to noise ratio at various concentrations of Etonogestrel in serum.

Concentration pg/ml	Percent Recovery	Matrix Effect	Intra-Assay Variability	Signal/Noise
25				8.7
50	87.5%	1.42	2.63%	28.6
100	77.6%	1.11	5.64%	56.6
250	83.4%	1.04	1.48%	149.4
500	92.8%	0.98	2.25%	170
1000	88.7%	1.01	3.86%	509.1

Table 2

Inter-assay accuracy, precision and signal to noise ratio at three different concentrations of Etonogestrel in serum.

	Predicted Concentration (pg/ml)	Measured Concentration (pg/ml)	Inter-Assay Accuracy	Inter-Assay Precision	Signal/Noise
Low QC	75	68.6	-8.5%	12.5%	36
Medium QC	750	691.2	-7.8%	13.0%	216.7
High QC	1750	1661.1	-5.1%	8.6%	237.8